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14. ABSTRACT
Our overall goal is to develop a safe and feasible model for the chemoprevention of a wide range of tobacco-related diseases. Our immediate goal, that is addressed over a 4-year study period, is to determine the effects of high tea consumption on biological markers of oxidative stress that mediate lung cancer risk. We are conducting a 6-month randomized, controlled, double-blinded chemopreventive trial in a group of COPD subjects who are being randomized to green or black tea preparations or a control intervention (matching placebo). Levels of 8-hydroxydeoxyguanosine and 8-F2-isoprostanes are used to measure DNA and lipid damage respectively. Changes in biomarkers of oxidative damage are being measured in urine, blood and exhaled breath condensate. The study protocol was approved by all parties in September 2003. Recruitment and screening of participants for eligibility criteria started in October 2003. By the end of December (2006), 275 participants signed the consent form and were screened for eligibility criteria (spirometry for lung function). A total of 262 subjects were eligible, however, 96 subjects dropped out during run-in and before randomization and 158 were randomized to one arm of the study. Currently 114 eligible subjects completed the study and 19 subjects are actively enrolled in the study. We expect that adherence to a regular pattern of tea is feasible and quantifiable among this high risk population.

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INTRODUCTION

Preventive strategies require identification of cancer-susceptible individuals resulting from combinations of carcinogen exposure and lack of protective factors. Oxidative reactions have been implicated as important modulators of human health and can play a role in both disease prevention and disease development. A large number of studies have demonstrated an increased oxidant burden and consequently increased markers of oxidative stress in the airspaces, breath, blood, and urine of smokers and of patients with chronic obstructive pulmonary disease (COPD) [1,2]. Changes in dietary habits with the intake of more cancer-chemopreventive agents appear to be a practical approach for cancer prevention in subjects with increased oxidative stress as is the case of subjects with COPD and ≥ 25 pack/year of smoking history.

The present study will investigate the ability of regular green and /or black tea consumption to decrease oxidative stress during the context of a randomized, controlled, double blinded, dietary intervention trial. Levels of 8-hydroxydeoxyguanosine (8-OHdG) will be used to measure DNA damage and levels of 8-F2 isoprostanes **(**8-epi-PGF2) and ethanes will be used to measure lipid damage**.** Testing for biomarkers of oxidative stress in exhaled breath condensate (EBC) will complement other innovative methods currently being investigated. The use of this novel strategy might enable further classification of people at risk of increased oxidative stress lung cancer, such as smokers, workers in nuclear weapons plants, Gulf War veterans [3], and US Marines by degree of risk. Such refinement of risk analysis might then be used to identify candidates for screening studies.

BODY

Task 1. **Preparation, protocol development and analysis of tea extracts and placebo (QC/QA) for tea polyphenols (Months 1-7)**

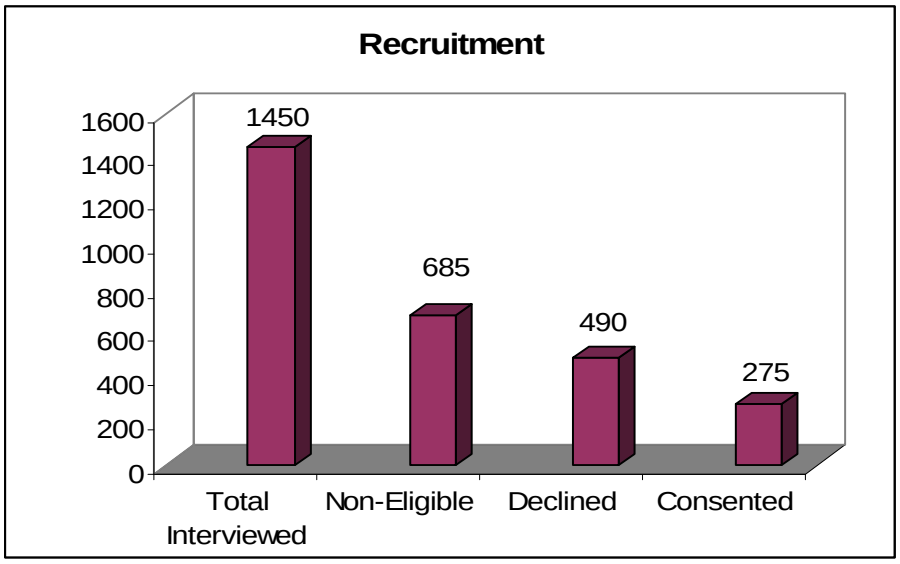
Completed: See previous reports

Task 2. **Recruitment/ eligibility, Run-In & baseline assessment of oxidative stress (Month 8-36)**

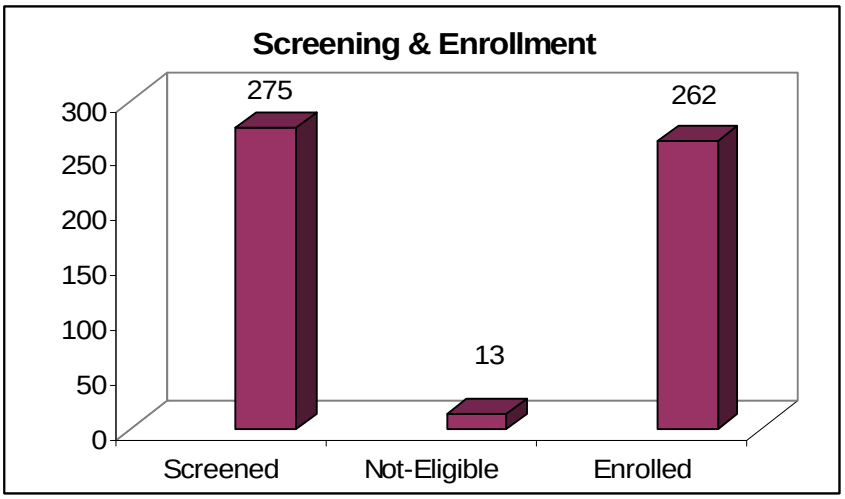
a & b) Potentially eligible subjects were recruited beginning in month 5 of the study and will be continuing through the first 6 months of year 5 .

During the last 2 years of recruitment, we found that we have to screen more than 1000 subjects to be able to find 200 eligible subjects and 40 % of the subjects are more likely to drop-out during run-in (before randomization) due to various reasons . Therefore, we are expending the recruitment time to 48 months to be able to have 150 subjects completing the study.

To date, 1450 subjects were interviewed by phone for eligibility criteria. 685 subjects were not eligible because of age, pack/year of cigarettes, medications, had cancer, or currently enrolled in another study. 490 subjects refused to participate (won't give up tea, cannot drink much tea, study too long).



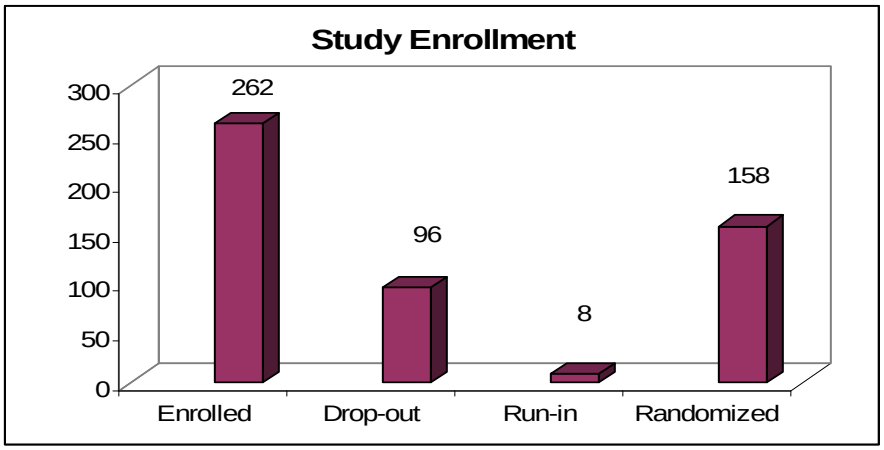
By the end of December (2006), 275 participants signed the consent form and were screened for confirmation of COPD eligibility criteria (spirometry for lung function tests). Thirteen subjects were not eligible and 262 subjects were enrolled in the study



c) <u>Eligible subjects will complete 1-month run-in period during which they will consume the placebo beverage and complete all baseline questionnaires</u>.

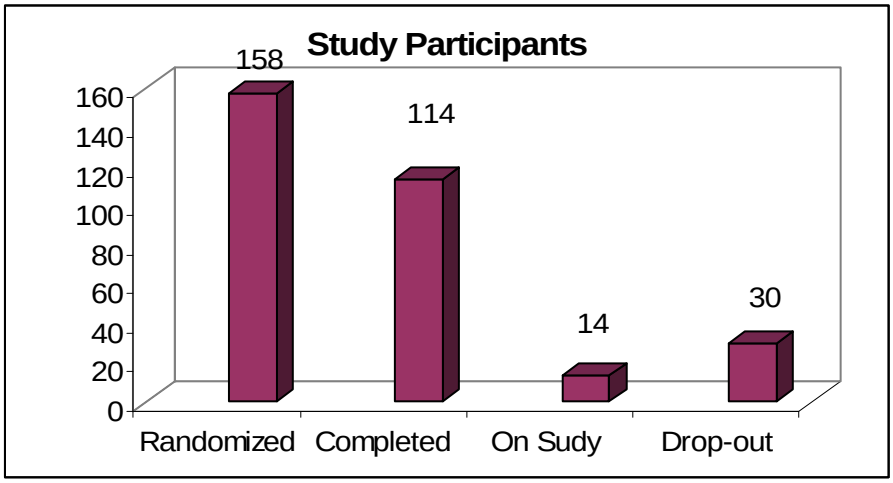
By the end of December (2006), 262 eligible subjects completed all baseline questionnaires and started the run-in period. Each enrolled participant, received 1-month of placebo tea bags, study teacup, a 3-minute timer, the monthly diary and health monitoring forms, and sterile urine cups. Subjects were contacted biweekly to ensure and encourage adherence and to monitor any adverse event.

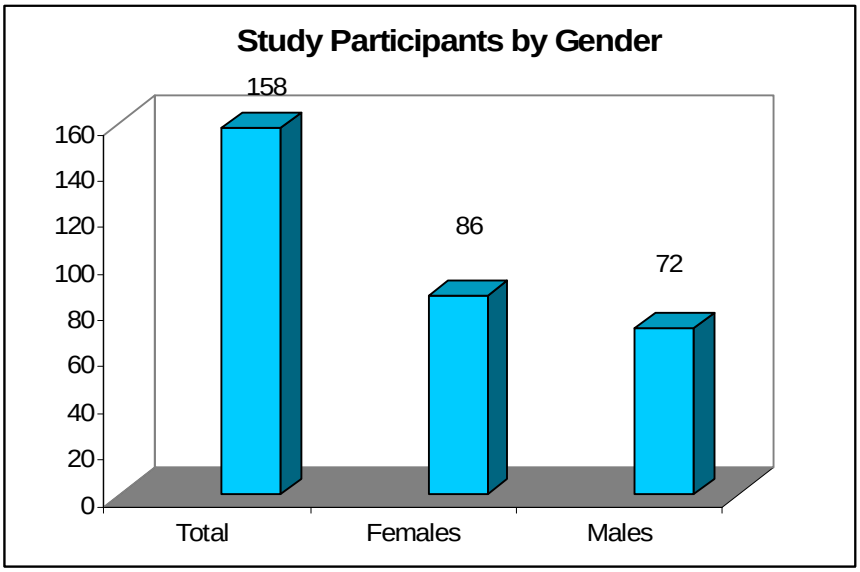
Ninety-six of the participants dropped-out from the study in the first week. The main reported causes of drop-out are: 1) could not stop coffee, do not like the taste of the tea, and caffeine intolerance. A total of 158 subjects completed the run-in and were randomized to one arm of the study.

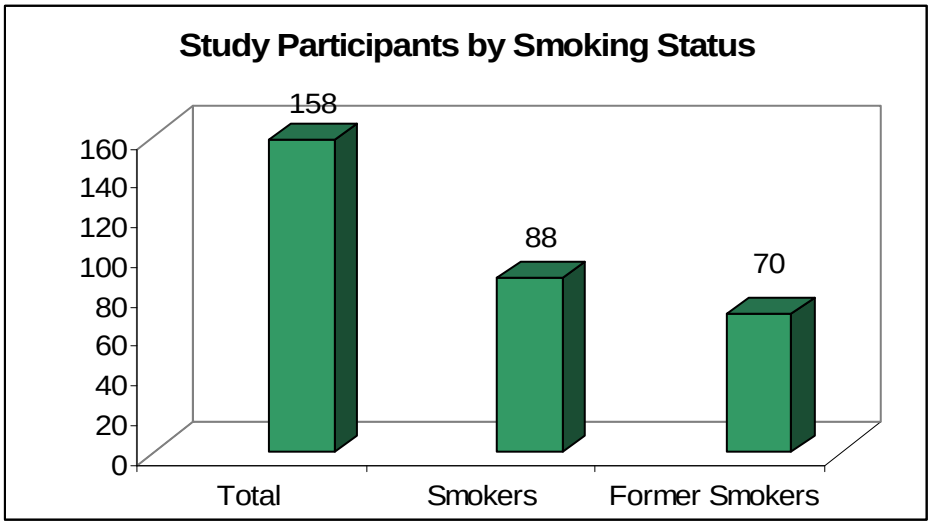


d & e) Subjects who complete the run-in period will provide blood, urine and exhaled breath condensate (EBC) samples for biomarker analysis. Subjects will be asked to provide buccal cells and induced sputum samples for storage.

To-date 158 subjects completed the run-in successfully and were randomized to one of the study arms: Green tea, black tea, or placebo. All randomized subjects provided blood and urine samples, exhaled breath condensate (EBC), and buccal cell samples.







Demographic data for the study participants per randomization group are provided in the following tables.

Table 1

			Treatment Groups	
	Overall	A	B	C
Age at Consent				
Mean	60.5	60.21	60.37	60.94
Std. Dev.	8.91	8.75	8.52	9.61
Var.	79.35	76.48	72.58	92.38
n	158	53	54	51

Table 2

			Treatment Groups	
	Overall	A	B	C
Pack-years				
Mean	46.39	49.47	46.34	43.25
Std. Dev.	21.47	22.66	21.08	20.54
Var.	460.76	513.42	444.23	421.88
n	158	53	54	51

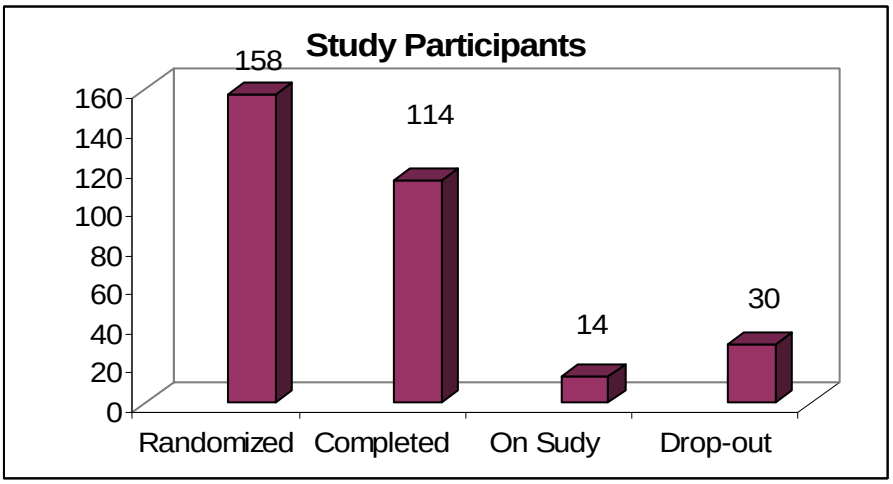
f) Determination of each subject's baseline history of smoking, diet and tea intake, plasma catechins, and levels biomarkers of oxidative stress at baseline.

All baseline data for enrolled participants were collected and all data was entered into the computer. Quality control of the data is performed regularly. This is an ongoing process with ongoing recruitment and enrollment.

Task 3. Intervention, Follow-up & Exit focus groups to study the effect of tea consumption on DNA (8-OHdG) and lipid (8-epi-PGF2) damage in blood, urine, and EBC (Months 10-43).

a) Randomize eligible COPD chronic and former smokers into one of three interventions: black tea, green tea or placebo for 6 months.

To-date 158 subjects have been randomized to 1 of the 3 arms of the study. One hundred and fourteen subjects have already completed the 6-month intervention study and 14 are currently completing the study.



b) To maintain high adherence to the study intervention including collection of blood, urinary, and EBC samples through the 6-month intervention period and 1-month follow-up period.

Study participants are contacted biweekly by phone to ensure adherence. Subjects complete a tea and smoking diary in which they report their daily intake of tea (amount and time) and the number of cigarettes smoked each day. They also complete a health monitoring form in which they report any change in medication use, any health-related event, or any perceived adverse event.

c) To identify issues affecting recruitment and retention of chronic and former smokers with COPD in a lung cancer prevention trial.

d) To determine whether subjects will continue to consume tea regularly after the end of the intervention.

Exit and satisfaction questionnaire were collected from all participants that completed the study. Data is being entered into the computer database. This is an ongoing process and final results will be available at the end of the study when randomization arm will be revealed. To date, the most common causes of drop-out are too much fluid and time commitment.

Task 4. **Laboratory analyses and data entry (Months 8-45)**

a) Quality control assurances of laboratory methods

We have completed all the validation and quality control measures for the biomarkers of oxidative stress. Our quality control and validation data show that the urinary biomarkers of oxidative DNA and lipid damage are stable even when left at room temperature for 3 consecutive days.

b) & c) Urinary and blood biomarkers' analyses and quality control

Laboratory analyses of urinary and blood biomarkers of oxidative damage started on time as scheduled. All laboratory analyses undergo quality control/quality assurance measures before being sent for data entry. This preliminary summary represents the data that had been entered into our database. This is an ongoing process.

Measurements of 8-hydroxy-2'-deoxyguanosine (8OHdG) in human urine and lymphocyte DNA by high performance liquid chromatography-electrospray tandem mass spectrometry

A method for quantification of 8OHdG in human urine by HPLC-tandem mass spectrometry has been implemented and validated in Dr. Chow's laboratory. The analysis is performed on a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometric system in tandem with a Surveyor LC system. The urine sample (50 μl) is diluted 1:1 with water and injected onto the HPLC system. HPLC separation is achieved with a BDS Hypersil C_{18} column (150 x 2.1 mm, 5μ) and a gradient mobile phase. The gradient starts at 1% methanol and 99% 10 mM ammonium formate and is increased linearly to 80% methanol and 20% ammonium formate by 15 minutes. The system is re-equilibrated with 1% methanol and 99% ammonium formate for 5 minutes before the next injection. The flow rate is 0.2 ml/min. 8OHdG (from precursor ion *m/z* 284 to product ion *m/z* 168) and 2'-deoxyguanosine (from precursor ion *m/z* 268 to product ion *m/z* 152) are detected with multiple reaction monitoring (MRM) in the positive ion mode utilizing electrospray ionization. Linear calibration curves have been established from 0.3 to 30 ng/ml (1-100 nM). The within-day and between-day coefficient of variation of the assay is less than 10%. 8OHdG is found to be stable in urine when stored at room temperature for 72 hours.

Dr. Chow's laboratory has also tested various DNA digestion procedures for measurement of 8OHdG levels in DNA to maximize release of normal nucleosides and 8OHdG and minimize oxidation of 2'-deoxyguanosine and DNA during sample preparation and handling. Dr. Chow's lab had optimized the procedures for isolating DNA from blood lymphocytes for 8OHdG measurements. All laboratory analyses are ongoing. Representative LC/MS/MS chromatograms for 8OHdG (*m/z* 284/168) have been presented before (See previous report).

Measurements of 8-isoprostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) in human urine by high performance liquid chromatography-electrospray tandem mass spectrometry

A method for quantification of 8-isoprostaglandin $F_{2\alpha}$ in human urine by HPLC-tandem mass spectrometry has been implemented and validated in Dr. Chow's laboratory. The analysis is performed on a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometric system in tandem with a Surveyor LC system. The urine sample (1 ml) is extracted with a solid phase extraction procedure before injecting onto the HPLC system. Isotope labeled 8-isoprostaglandin $F_{2\alpha}$-D4 (8-iso-$PGF_{2\alpha}$-D4) is used as the internal standard. HPLC separation is achieved with a BDS Hypersil C_{18} column (150 x 2.1 mm, 5μ) and a gradient mobile phase consisting of 2 mM ammonium acetate (A) and 5:95 methanol:acetonitrile (B). The gradient starts at 20% B and increases linearly to 35% B by 27 minutes. The system is re-equilibrated with 20% B for

10 minutes prior to the next injection. Flow rate is 0.2 ml/min. 8-iso-$PGF_{2\alpha}$ (from precursor ion *m/z* 353 to product ion *m/z* 193), 8-iso-$PGF_{2\alpha}$-D4 (from precursor ion *m/z* 357 to product ion *m/z* 197), and prostaglandin $F_{2\alpha}$ (from precursor ion *m/z* 357 to product ion *m/z* 197) are detected with multiple reaction monitoring (MRM) in the positive ion mode utilizing electrospray ionization. Linear calibration curves have been established from 20 to 5000 pg/ml. The within-day and between-day coefficient of variation of the assay is less than 10%. Representative chromatograms for *(8-iso-$PGF_{2\alpha}$)* have been presented before (See previous report).

We have completed the urinary analyses of biomarkers of oxidative DNA damage (8-OHdG) and lipid damage (8-F2 isoprostanes), and creatinine for the first and second group of subjects who completed the 6-month study. Biomarkers were measured at baseline, month 3 (mid-intervention), and month 6 (end of intervention). Because of the nature of the study (randomized and blinded), we will not be able to sort the data by randomization group until the end of the study, and hence, we will not be able to comment on the effect of tea drinking until the end of the study. Summary of the overall entered data is presented in the Table below.

8-OHdG Urine (ng 8-OHdG/mg Creatinine)	
Mean	4.056997892
Standard Error	0.129144973
Median	3.541513514
Standard Deviation	2.718187243
Sample Variance	7.388541887
Range	27.17706977
Minimum	0.034
Maximum	27.21106977
Sum	1797.250066
Count	443
Confidence Level(95.0%)	0.253814465

Total EGC Catechin Evening Urine (ng/mL)	
Mean	1997.757901
Standard Error	489.4951
Median	316.47
Standard Deviation	4405.4559
Sample Variance	19408041.69
Range	20004.1
Minimum	8.16
Maximum	20012.26
Sum	161818.39
Number of PATS w/ measurable data increase compared to V0	81
Confidence Level(95.0%)	974.1272409

d) Oxidative stress biomarkers in exhaled breath condensate

Measurements of 8-isoprostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) in human exhaled breath condensate

A commercially available enzyme immuno assay kit (Cayman Chemical, Catalog No. 516351) which has shown a limit of quantification of 4 pg/ml for measurements of 8-iso- $PGF_{2\alpha}$ levels in the breath condensate samples. We have established a reproducible 8-iso-$PGF_{2\alpha}$ calibration curve from 3.91 to 500 pg/ml. The between-day and within-day coefficient of variance is less than 11%. A number of baseline exhaled breath condensate samples were tested using this procedure and the 8-iso-$PGF_{2\alpha}$ levels were found to be at the low end of the calibration curve (2-4 pg/ml). With a concentrating factor of 10, we were able to observe absorbance readings comparable to those observed with concentrations of 20-30 pg/ml. Because this is a more reliable concentration range to monitor any modulating effects from tea intervention, we plan to concentrate all exhaled breath condensate samples by a factor of 10 prior to sample analysis. Exhaled breath condensate samples are currently being analyzed.

Measurements of Nitric Oxide (NO) and Ethane in Exhaled Air

Measurements of NO and ethane in exhaled air is being done at baseline and month 6 (end of intervention). All laboratory analyses undergo quality control/quality assurance measures before being sent for data entry. This preliminary summary represents the data that had been entered into our database. This is an ongoing process. Summary of the overall entered data is presented in the Table below.

Visit	Nitric Oxide (ppb)	Carbon Monoxide (ppm)	Ethane (ppb)
	Range	Range	Range
Baseline	5.2 – 77.0	0 – 52.0	0.4 – 14.8
Month 6	8.1 – 85.0	0.3 – 32	0.5 – 21.4

Antioxidant levels in blood

We have completed the analyses of blood antioxidants for the first 2 groups of participants who completed the study. Data entry and laboratory analyses for the third cohort is ongoing

Antioxidant Enzymes Final Data

DOD	CAT (nmol/min/g Hb)	GPx (nmol/min/g Hb)	SOD (U/g Hb)
Mean	597,248.98	26,585.17	5,366.94
Standard Error	10,804.46	549.13	108.93
Median	578,872.52	24,729.50	5,041.89
Standard Deviation	174,551.37	9,286.64	1,842.10
Sample Variance	30,468,179,876.48	86,241,725.00	3,393,341.62
Range	1,258,631.88	57,483.55	10,365.73
Minimum	209,148.44	9,711.17	1,643.06
Maximum	1,467,780.32	67,194.73	12,008.79
Sum	155,881,983.83	7,603,357.63	1,534,944.93
Count	261.00	286.00	286.00
Confidence Level(95.0%)	21,275.37	1,080.87	214.40

Development of the methodology for RNA extraction from sputum

We have successfully developed the methodology for RNA extraction from sputum with a significant yield of RNA. Preliminary testing of gene expression of proliferation and apoptosis are successful.

KEY RESEARCH ACCOMPLISHMENTS

- Development and approval of the study protocol
- Development and approval of all study forms and questionnaires
- Ongoing Successful recruitment and screening
- Ongoing Successful enrollment in the study
- Ongoing Successful collection of biological samples (blood, urine, EBC, buccal and sputum samples**)**
- Validation and quality control of all laboratory methods
- Ongoing laboratory analyses of biological samples.
- One hundred and fourteen participants successfully completed the study.
- Fourteen participants are currently completing the study
- Eight participants started the run-in
- Ten new participants scheduled for clinical eligibility in the next 2 weeks
- Successful development of methodology for RNA extraction from sputum.
- Preliminary analyses on RNA gene expression in sputum samples

REPORTABLE OUTCOMES

A Poster was submitted to Military Health Research Forum in Puerto Rico (April 30- May 4, 2006)

CONCLUSIONS

During the last 4 years of the study, we were able to reach a large number of potential participants. We interviewed (initial screening) 1450 subjects and randomized 158 eligible subjects in the study. Identification of eligible participants is quite a challenge. however, we were successful in reaching a large pool of potential subjects. Interviewing, initial screening, and enrollment are ongoing. We have 10 new subjects scheduled during the next 2 weeks and we are planning to maximize our recruitment efforts to enroll and randomize the last 22 participants during the first half of this coming year.

Because tea is one of the most popular beverages consumed worldwide, the relationship between tea consumption and human cancer incidence is an important concern. Tea can be easily consumed with one's ordinary meals making compliance and adherence to dietary intervention more likely to succeed. Thus, the role of tea drinking as a potential inhibitor of carcinogenesis merits careful evaluation. We believe that a program of nutritional intervention with realistic dietary modifications that are effective, safe, and acceptable should be the cornerstone of any cancer prevention strategy.

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APPENDICES

1. Abstract entitled" A Chemoprevention Trial To Study The Effects Of High Tea Consumption On Smoking-Related Oxidative Stress"

A CHEMOPREVENTION TRIAL TO STUDY THE EFFECTS OF HIGH TEA CONSUMPTION ON SMOKING-RELATED OXIDATIVE STRESS

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BACKGROUND/PURPOSE: Oxidative reactions have been implicated as important modulators of human health and can play a role in both disease prevention and disease development. A large number of studies have demonstrated an increased oxidant burden and consequently increased markers of oxidative stress in the airspaces, breath, blood, and urine of smokers and of patients with chronic obstructive pulmonary disease (COPD). The overall goal of this study is to develop a safe and feasible clinical research approach that will serve as a model for the chemoprevention of a wide range of tobacco-related diseases. Our immediate goal, that is addressed over a 4-year study period, is to determine the effects of high tea consumption on biological markers of oxidative stress that mediate lung cancer risk, including, 8-hydroxydeoxyguanosine **(**8-OhDG), F2-isoprostanes (8-epi-PGF2), ethanes, and nitric oxide. We will also determine if high tea consumption can modulate the genes involved in the carcinogenic process in damaged bronchoepithelial cells**. METHODS**: We are conducting a 6-month randomized, controlled, double-blinded chemopreventive trial in a group of COPD subjects (FEV1 ≤ 85% of the standard) with 25 or more pack-years of smoking history. The participants are stratified on smoking status (current or former) and gender, and are being randomized to green or black tea preparations or a control intervention (matching placebo). Levels of 8-OHdG are used to measure DNA damage and levels of 8-epi-PGF2 and ethanes are used to measure lipid damage. Changes in biomarkers of oxidative damage are measured in urine, blood and exhaled breath condensate. Changes in the gene expression of biomarkers of proliferation (EGFR, JUN, FOS, Ki-67) and apoptosis (caspase 3) in induced sputum are being assessed. **RESULTS:** The study protocol was approved by all parties in September 2003. Recruitment and screening of participants for eligibility criteria started in October 2003. To date, 110 subjects have been enrolled in the study and 80 have already completed the study. We have completed the urinary analyses of biomarkers of oxidative and the RNA gene expression and modulation for the first group of subjects who completed the 6-month study. Biomarkers of oxidative stress were measured at baseline, month 3 (mid-intervention), and month 6 (end of intervention) while gene expression was measured at baseline and end of the study. Summary of the overall entered data will be presented. **CONCLUSION**: We expect that adherence to a regular pattern of tea is feasible and quantifiable among this high risk population**.**